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Detection and Quantification of Virus DNA in Plasma of Patients with Epstein-Barr Virus-Associated Diseases

MITSUAKI YAMAMOTO,[1]* HIROSHI KIMURA,[1] TAKASHI HIRONAKA,[2] KANJI HIRAI,[2] SHINJI HASEGAWA,[1] KIYOTAKA KUZUSHIMA,[1] MOTOHIRO SHIBATA,[1] AND TSUNEO MORISHIMA[1]

Department of Pediatrics, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466,[1] and Department of Cell Regulation, Division of Virology and Immunology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113,[2] Japan

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Epstein-Barr virus (EBV) causes various diseases, such as infectious mononucleosis (IM), fatal IM, EBV-associated hemophagocytic syndrome (EBVAHS), and chronic active EBV infection (CAEBV). In the present study, cell-free EBV DNA was detected in the plasma of patients with EBV-associated diseases by PCR assay. The patients included 20 patients with IM, 2 patients with fatal IM, 4 patients with EBVAHS, 4 patients with CAEBV, and 38 healthy children (20 EBV seropositive and 18 EBV seronegative). In patients with IM, plasma samples were positive for EBV DNA in all patients (100%) in the acute phase and in 44% of the patients in the convalescent phase, but plasma samples from the 38 healthy control children were negative (0%) for EBV DNA. Quantitative PCR assay revealed that plasma from patients with IM contained the highest amount of virus DNA within 7 days following the onset of disease (mean, 6×10^4 copies per ml). The EBV DNA concentration decreased thereafter as the patients recovered. Plasma from patients with fatal IM contained more than 100 times more copies of EBV DNA (3×10^7 copies per ml) than plasma from patients with IM. Plasma from patients with the acute phase of EBVAHS contained 10 times more copies of EBV DNA (5×10^5 copies per ml) than plasma from IM, and then patients with the number of copies decreased similarly in both groups of patients in the convalescent phase (2×10^4 copies per ml). The amount of virus DNA in patients with CAEBV (6×10^4 copies per ml) was similar to that noted in patients with IM; however, it became higher (1×10^6 copies per ml) when the patients' clinical status deteriorated. These data suggest that the presence of cell-free EBV DNA in plasma is a common phenomenon in patients with EBV-associated diseases. The concentration of EBV DNA in plasma seems to be higher in patients with the more severe clinical categories of EBV diseases.

Epstein-Barr virus (EBV), which is ubiquitous in humans, is a causative agent of infectious mononucleosis (IM), fatal IM, EBV-associated hemophagocytic syndrome (EBVAHS), chronic active EBV infection (CAEBV), and lymphoproliferative disorders (4, 14–16, 18). Primary EBV infection in infants and younger children rarely manifests as typical IM, which is a self-limiting disease (4, 24). The last four diseases are usually severe and potentially fatal (10, 14–16). Besides, in patients with immunodeficiency such as patients with severe combined immunodeficiency and bone marrow transplant recipients, EBV could cause life-threatening diseases such as fatal IM or B-cell lymphoproliferative disorders (14, 16).

Virus DNA in the peripheral blood lymphocytes of patients with IM has been detected by PCR assay (26). Virus DNA was detected in the peripheral blood lymphocytes of between 54 and 94% EBV-seropositive healthy individuals (7, 27), since latently infected lymphocytes are present in about 1 in 10^6 lymphocytes (19). Virus DNA in mononuclear cells (MNCs) of patients with lymphoproliferative disease was also detected and quantified as a useful marker for monitoring patients and predicting the progression of the disease (17). Recently, cell-free EBV DNA also has been detected in the serum of patients with IM (5). As Spector et al. (23) showed in patients with human cytomegalovirus (HCMV) disease with human immunodeficiency virus infection, determination of cell-free virus DNA could be important in evaluating the progression of infections with herpesviruses. In the present study, EBV DNA in plasma was assayed quantitatively during the acute and convalescent phases of IM. We analyzed the relation between the amount of virus DNA and the course of the disease. Then, we also determined the amount of virus DNA in the plasma of patients with other EBV-associated diseases, including EBVAHS, fatal IM, and CAEBV in order to clarify the relationship between the quantity of virus DNA in plasma and the severity of disease in each clinical category.

MATERIALS AND METHODS

Patients and controls. Twenty patients with IM (11 males and 9 females; ages, 1 to 19 years), 4 children with EBVAHS (3 males and 1 female; ages, 2 to 4 years), 2 children with fatal IM (2 males; ages, 2 and 6 years), 4 children with CAEBV (4 males; ages 3 to 11 years), 20 EBV-seropositive healthy children (11 males and 8 females; ages, 1 to 14 years), and 18 seronegative healthy children (11 males and 7 females; ages, 0 to 5 years) were examined.

The patients with IM showed clinical symptoms such as fever, lymphadenopathy, hepatosplenomegaly, and skin rash (24). The diagnosis was made on the basis of serological examinations, elevated antibody levels to viral capsid antigen and early antigen, and a negative result for EBV nuclear antigen antibody (25). The patients with fatal IM had a high fever, skin rash, lymphadenopathy, and hepatosplenomegaly, with no family history of fatal IM. They showed a rapid progression of disease until liver failure and coagulopathy appeared, and they finally died within a few weeks (14). The patients with EBVAHS manifested a high fever and hepatosplenomegaly, with no malignancy or family history of this disease. The diagnosis was made on the basis of laboratory findings including pancytopenia, liver dysfunction, coagulopathy, elevated EBV antibody levels, and histiocytic hyperplasia with prominent hemophagocytosis in bone marrow (18). The patients with CAEBV showed intermittent or persistent fever over 6 months with lymphadenopathy and hepatosplenomegaly. The laboratory findings showed anemia, polyclonal gammopathy, and remarkably elevated EBV antibody levels. EBV genomes were detected in the affected tissues (15). Plasma

* Corresponding author. Phone: 8152 741-2111, ext. 2262. Fax: 8152 731-6137.

samples were obtained when the patients were diagnosed as having CAEBV, and two more samples were obtained during the course of deterioration.

Samples. Peripheral blood samples from the patients were collected in standard blood tubes containing EDTA anticoagulant. Blood samples were obtained with permission from seropositive and seronegative healthy children and were used as controls. Following centrifugation at 500 × *g*, plasma samples were collected. MNCs were isolated by Ficoll-Paque (Pharmacia, Piscataway, N.J.) density centrifugation. MNCs (10^6) were placed in 100 μl of Tris-EDTA (10 mM Tris-HCl, 10 mM EDTA) solution and were stored at −80°C. The samples were incubated in 0.6% sodium dodecyl sulfate (SDS) solution with proteinase K at 55°C for 2 h and were then extracted with phenol-chloroform; this was followed by ethanol precipitation. The extracted DNA was placed in Tris-EDTA (10 mM Tris-HCl, 0.5 mM EDTA) solution and was used for PCR.

Primers. Primer pairs specific for a 134-bp region were generated from the *Bam*HI-W region (positions 1544 to 1568 and positions 1653 to 1677 for 5′ and 3′ primers, respectively) of the EBV genome (9). The *Bam*HI-W region is highly conserved among the different EBV strains (22). The oligonucleotide probe was chosen from the sequences bounded by the primers (positions 1593 to 1632). This oligonucleotide was covalently cross-linked to alkaline phosphatase (8) by Iatron Laboratories, Chiba, Japan. For the quantitative PCR assay, the oligonucleotide probe was labeled with [γ-^{32}P]ATP.

PCR. PCR was performed under stringent conditions. To avoid false-positive results, we used fitted latex gloves, sterile pipette tips and tubes, aliquots of sterile reagents, and a separate set of pipettors in a separate room for the PCR sample handling (12).

DNA samples were boiled for 10 min and were immediately cooled on ice. One 50-μl sample of the reaction mixture consisted of PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 10 mmol each of the deoxynucleoside triphosphates, 20 pmol of each primer, 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and DNA solution from 10 μl of plasma or 500 ng of DNA from MNCs. Amplifications were carried out with a DNA thermal cycler (Perkin-Elmer Cetus) for 30 cycles. A single cycle consisted of denaturation (94°C for 1 min), annealing (60°C for 2 min), and primer extension (72°C for 3 min); this was followed by a 30-min extension period at 72°C in the final cycle.

Agarose gel electrophoresis and hybridization of labeled probe. The amplified products were electrophoresed in 1.5% agarose gel and were subsequently transferred onto a nylon filter membrane, with 10× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) used as the transfer buffer. The nylon filter membranes were prehybridized in hybridization buffer containing 0.1% sarcosine, 0.2% SDS, 5% blocking reagent (Boehringer-Mannheim, Mannheim, Germany), 30% formamide, and 5× SSC at 37°C for 1 h and then were hybridized with an oligonucleotide-alkaline phosphatase conjugate in the hybridization buffer at 37°C overnight. The filter membranes were washed three times with washing buffer containing 2× SSC and 0.1 mM $ZnCl_2$ at 42°C for 30 min and then were incubated in the washing buffer with 0.1% SDS at 42°C for 45 min. The filter membranes were developed in nitroblue tetrazolium and 0.2 mg of 5-bromo-4-chloro-3-indoyl phosphate per ml in 10 ml of 0.1 M Tris-HCl (pH 9.5)–0.1 M NaCl–10 mM $MgCl_2$ for 10 h for visualization (28).

Sensitivity and specificity of the PCR assay. To estimate the sensitivity of the PCR assay, serial 10-fold dilutions of plasmid DNA containing the *Bam*HI-W region were prepared and amplified by the PCR assay. The amplified DNA was detected with alkaline phosphatase-labeled probes and Southern hybridization. We were able to detect 10 or more copies of the plasmid DNA. To estimate the specificity of the PCR assay, herpes simplex virus types 1 and 2, HCMV, human herpesvirus 6, and varicella-zoster virus DNAs were also purified, amplified, and measured by the PCR assay. No specific amplification of any virus DNA other than that of EBV was noted.

In order to exclude the possibility that the detected virus DNA was associated with MNCs, fresh plasma specimens from three patients with IM were filtrated through a 0.45-μm-pore-size filter prior to preparation of the DNA. These samples were then centrifuged at 7,000 × *g* to remove agglutinated virus DNA. The supernatant was then centrifuged at 70,000 × *g* for 2 h to examine whether the virus DNA could be pelleted. Under these conditions virus particles were expected to be pelleted (6).

Quantitative PCR assay. The amplified DNA was subjected to electrophoresis and was transferred onto a nylon membrane and was then hybridized with a ^{32}P-labeled probe. Southern hybridization was accomplished in hybridization buffer containing 2× SSC and 0.1% SDS with Denhardt's reagent at 55°C overnight. Hybridized membranes were washed in 2× SSC with 0.05% SDS at 45°C three times for 30 min each time. The radioactivity of the PCR products in the Southern blots was then measured with the BAS 2000 system (Fuji Photo Film Co., Tokyo, Japan) (1, 11). The standard curve was prepared by using serial 10-fold dilutions of the plasmid DNA solution, and the plasmid DNA was amplified in the same assay (Fig. 1) (1, 21).

Data analysis. Fisher's exact test was used in the statistical analysis. *P* values of less than 0.05 were considered statistically significant.

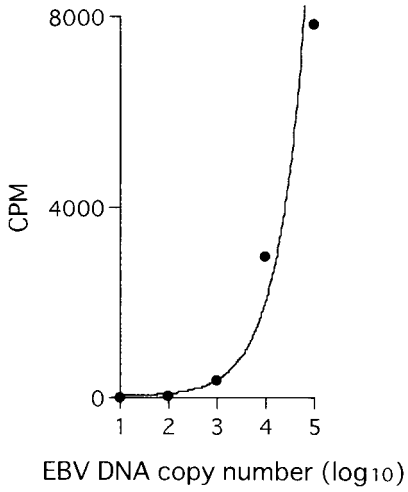

FIG. 1. Standard curve for quantitative PCR analysis of EBV. Counts per minute are plotted as a function of the logarithm of the number of DNA copies for serial 10-fold dilutions of plasmid DNA.

RESULTS

Detection of EBV DNA from plasma and MNCs. Plasma samples from 20 patients with IM and 38 healthy children were examined (Table 1). Virus DNA was detected in the plasma of all patients (13 of 13; 100%) with IM during the acute phase (within 14 days of disease onset). During the convalescent phase (after day 15), virus DNA was detected in the plasma of four of nine patients (44%). Virus DNA was not detected in the plasma of any of the seropositive or seronegative healthy controls. Virus DNA was detected in MNCs from all patients with IM (100%), in 9 of 20 seropositive controls (45%), and in none of the 18 seronegative controls.

Because EBV DNA was detected in the three filtrated plasma samples, the virus DNA detected was not a result of contamination of MNCs. Then, we determined whether the virus DNA detected in the plasma was originated from cell-free particles or DNA fragments of EBV. In this experiment EBV DNA was detected from both the pellet and the supernatant (data not shown). This implied that both virus particles and DNA fragments of EBV were present in plasma.

Quantitative analysis of plasma EBV in patients with IM. We performed Southern hybridization using serial samples from three patients with IM (Fig. 2). Corresponding to the clinical improvement, the intensity of hybridization decreased

TABLE 1. EBV DNA in plasma and MNC samples

Subject	No. of samples positive/total no. of samples (%)	
	Plasma	MNCs
Patients with IM at[a]:		
0–14 days (n = 13)	13/13 (100)[b]	13/13 (100)
15–40 days (n = 9)	4/9 (44)[b]	9/9 (100)
Healthy children		
EBV seropositive (n = 20)	0/20 (0)[b]	9/20 (45)
EBV seronegative (n = 18)	0/18 (0)	0/18 (0)

[a] Thirteen samples were obtained in the acute phase (0 to 14 days) and 9 samples were obtained in the convalescent phase (15 to 40 days) from 20 patients with IM.

[b] $P < 0.01$.

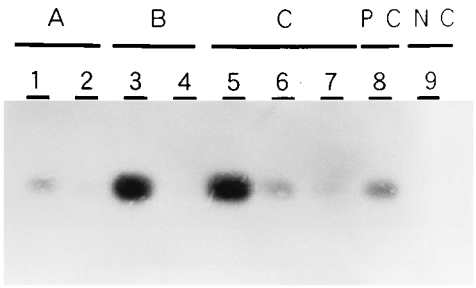

FIG. 2. Southern blot hybridization of serial plasma samples from three patients with IM. Lanes 1 and 2 (patient A), days 4 and 14 of disease onset, respectively; lanes 3 and 4 (patient B), days 17 and 25 of disease onset, respectively; lanes 5, 6, and 7 (patient C), days 17, 25, and 37 of disease onset, respectively; lane 8 (PC), a positive control; lane 9 (NC), a negative control.

gradually. The first samples were positive with a stronger density, and then those of the bands decreased in the following second and third samples, suggesting that the amount of virus DNA decreased during the course of infection. Therefore, the number of virus DNA copies in plasma was determined by quantitative PCR assay. We analyzed 27 samples from 20 patients with IM (Fig. 3). Patients with IM had the largest number of virus DNA copies within 7 days following disease onset, with a mean titer of 6×10^4 copies per ml. The mean titer was 10^4 copies per ml in the second week. The number of virus copies declined to less than 10^3 copies per ml in 6 of 12 samples after day 15 as the patients recovered. A patient whose clinical symptoms were severe and prolonged remained positive for EBV DNA on day 37.

Quantitative analysis of plasma from patients with various EBV diseases. From an analysis of the amount of EBV DNA in the plasma of patients with various EBV-associated diseases, including EBVAHS, fatal IM, and CAEBV (Fig. 4), the plasma of patients with EBVAHS showed a mean titer of 5×10^5 copies per ml in the acute phase, which was 10 times higher than that in patients with IM. The DNA copy number decreased in the convalescent phase, with a mean titer of 2×10^4 copies per ml. A mean titer of 3×10^7 copies per ml was found in the plasma of both of the patients with fatal IM; this was more than 100 times higher than that in the plasma of patients with IM. In the plasma of patients with CAEBV, virus DNA was present at a mean titer of 6×10^4 copies per ml, which was similar to that noted the plasma of patients with IM. The plasma of two patients had a higher titer (10^6 copies per ml) when their clinical status deteriorated (Fig. 4).

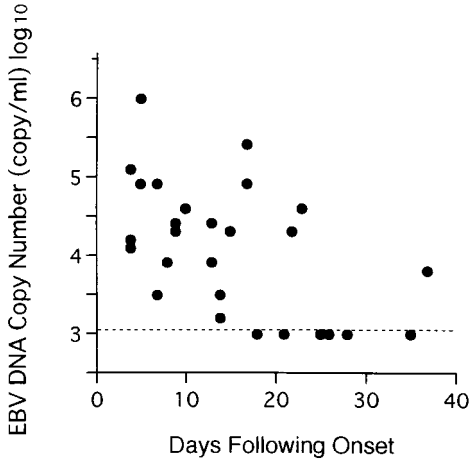

FIG. 3. Quantification of EBV DNA in plasma from 27 samples obtained from 20 patients with IM. The sensitivity of the PCR assay (dashed line) was <1,000 copies per ml.

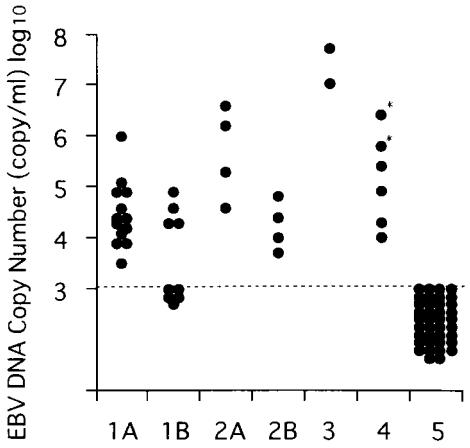

FIG. 4. Quantification of EBV DNA in plasma of patients with various EBV-associated diseases. 1A, acute phase of IM (n = 13); 1B, convalescent phase of IM (n = 9); 2A, acute phase of EBVAHS (n = 4); 2B, convalescent phase of EBVAHS (n = 4); 3, fatal IM (n = 2); 4, CAEBV (n = 4); 5, healthy individuals including EBV-seropositive and EBV-seronegative children (n = 38). Two patients with CAEBV had a higher EBV DNA titer when their clinical state deteriorated (*). The dashed line indicates the sensitivity of the PCR assay.

DISCUSSION

In the present study, we used PCR to detect EBV DNA in plasma and found that patients with various EBV-associated diseases were positive for EBV DNA. In patients with IM the positive ratio was 100% during the acute phase and decreased to 44% in the convalescent phase, whereas no EBV DNA was detected in either EBV-seropositive or EBV-seronegative healthy controls. The EBV DNA in plasma seemed to be derived from both viral particles and free DNA fragments. Recently, Gan et al. (5) reported the detection of cell-free EBV DNA in the serum of two patients with acute IM. We confirmed the result in 20 patients and found that the presence of EBV DNA in plasma is a common phenomenon in patients with IM. As shown in patients with HCMV infections (23), the presence of cell-free virus DNA may be a good marker of active virus infection with herpesviruses.

Quantitative PCR has proven useful for monitoring the progression of human immunodeficiency virus, hepatitis C virus, varicella-zoster virus and HCMV disease and for estimating the efficacy of therapeutic agents in patients with human immunodeficiency virus, hepatitis C virus and HCMV infections (2, 3, 13, 20, 21). We also reported the usefulness of quantitative PCR analysis for diagnosis and estimating the efficacy of therapeutic agents in patients with herpes simplex virus infection (1). In patients infected with EBV manifesting as IM, the decrease in the number of virus-infected peripheral blood lymphocytes correlates with clinical improvement (19). Our study of quantitative PCR showed a decrease in EBV DNA levels in plasma corresponding to clinical improvement in patients with IM. Thus, the concentration of EBV DNA in plasma seems to be correlated with the clinical status of patients with IM.

Quantitative analysis of cell-free EBV DNA in plasma was also applied to patients with other severe EBV-associated dis-

eases. The number of virus DNA copies in plasma samples from patients with fatal IM was more than 100 times higher than the average copy number in patients with nonfatal IM, suggesting that far more virus replication occurred in patients with fatal IM. EBVAHS is a nonneoplastic, generalized histiocytic proliferation with marked hemophagocytosis associated with a systemic viral infection. In a course of disease lasting several weeks to months, patients have fever, hepatosplenomegaly, pancytopenia, liver dysfunction, coagulopathy, and histiocytic hyperplasia with prominent hemophagocytosis in bone marrow (18). Virus DNA levels in the patients with acute-phase EBVAHS were 10 times higher than those in patients with IM. The amount of virus DNA in patients with EBVAHS similarly decreased in the convalescent phase. Patients with CAEBV often develop life-threatening complications over the course of months to several years (15). The clinical features are intermittent or persistent fever, lymphadenopathy, hepatosplenomegaly, and a tendency to pancytopenia and polyclonal gammopathy (15). Virus DNA copy numbers in the plasma of four patients with CAEBV were almost the same as the numbers in the plasma of patients in the acute phase of IM. However, two patients had higher titers of EBV DNA when their clinical status deteriorated. This suggested that the viral burden was related to the clinical signs and symptoms of CAEBV.

In conclusion, our results show that the presence of cell-free EBV DNA in plasma is a common phenomenon in patients with EBV-associated diseases and that quantitative analysis is useful for evaluating the severity of disease and the clinical status of patients with such diseases. Quantitative PCR could also prove to be useful for evaluating the efficacy of therapeutic agents in patients with EBV-associated diseases in the future.

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